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Detection of *Coxiella burnetii* and sequencing the IS1111 gene fragment in bulk tank milk of dairy herds

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Abstract

Coxiella burnetii is the etiologic agent of Q fever in human. This study is aimed to determine the contamination rate of *C. burnetii* in bulk tank milk samples. In this study, a total number of 100 bulk milk samples collected from dairy farms in northeast of Iran and were examined for *C. burnetii* using touchdown PCR assay. The primers were designed to amplify a 687-bp fragment of the IS1111 gene of *C. burnetii*. Five samples (5%) of bovine milk were found positive for *C. burnetii*. Sequence analysis of PCR products revealed 100% identity with published sequences of *C. burnetii* in genBank. Results of this study indicate that bovine milk is an important source of *C. burnetii* infection in Iran.

Keywords*:* Bulk tank milk, *Coxiella burnetii*, Touchdown PCR

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Introduction

Coxiella burnetii is an obligate intracellular, gram-negative coccobacillus and the agent of Q fever in human. *C. burnetii* is also highly infectious, with a dose of 1 to 10 organisms capable of causing Q fever in humans (Derrick, 1937). These unique feature of *C. burnetii*, along with its aerosol route of transmission, led to considering it as a potential weapon for bioterrorism (Franz *et al.,* 1997, Kim *et al*, 2005). This microorganism has been classified in biosafety level 3 of microbial agents (CDC, 2000-1). *C. burnetii*, is one of the zoonotic agents that widely distributed and has been reported from most countries except New Zealand (Fournier and Raoult, 2003). Ruminants are the main reservoir of infection for human (Baca and Paretsky, 1983). Ticks are the primary reservoir of *C. burnetii* and responsible for transmission to wild and domestic animals (Pinskey *et al*., 1991). The organism is shed via urine, feces, and milk of infected animals and has a particularly high concentration during parturition (Abinanti *et al*., 1953). Although human become infected usually by inhalation of aerosols contaminated with birth fluid, placenta, urine and feces of infected animals, but oral transmission by ingestion of contaminated raw milk or dairy products could lead to Q fever (Rodolakis *et al*., 2007), and contaminated milk has induced sero-conversion in human volunteers without clinical signs (Honarmand, 2012). Although animals do not often show typical clinical symptoms, but infection might be associated with abortion (particularly in sheep and goats and rarely in cows) and reproductive disorders (Ormsbee *et al*., 1978). In human, the disease is most often asymptomatic or with fairly nonspecific symptoms such as high fever, headache, myalgia, cough, and fatigue (about 60% of infections), but acute or chronic forms can also occur (Arricau-Bouvery and Rodolakis, 2005, Barlow *et al*., 2008, Zhang *et al*., 1998). Previously, the diagnosis of Q fever infection was mainly based on serological methods, and the most commonly used method was immunofluorescence assay (Field *et al*., 2000). Since this organism is an obligate intracellular organism, it cannot be cultured on routine bacteriologic media (Fournier *et al*., 1998). Isolation of *C. burnetii* is not performed for routine diagnosis, because it is difficult, time consuming and requires biosafety level 3 laboratories (Norrung *et al*., 1991). PCR is a highly sensitive and specific detection method that has been recently used to trace *C. burnetii* in clinical samples (Muramatsu *et al*., 1996, Muramatsu *et al*., 1997). This method has also been used for screening (Kim *et al*., 2005, Ongor *et al*., 2004) and determining the presence of the bacteria in milk, feces or vaginal swaps (Berri *et al*., 2000). Recently a PCR assay with primers based on, repetitive, transposonlike element (Trans-PCR) proved to be highly specific and sensitive (Willems *et al*., 1994). The objective of the present study was to determine the presence of *C. burnetii* in bulk tank milk, in northeast of Iran using touch-down PCR assay.

Material and methods

Sample collection

One hundred bulk tank milk samples from dairy farms in northeast of Iran were collected in 50 ml sterile Falcon tubes, and immediately transferred to laboratory at 4°C using ice packs and stored at -20°C until use.

DNA extraction

The DNA from milk samples were extracted using the First-Magnetic Milk Kit (Gen-ial, Germany) protocol as indicated by manufacturer. The DNA was quantified using spectrophotometer (Ultraspec 2000, Pharmacia Biotech) and diluted to 50 ng mL^{-1}, the extracted genomic DNA stored at -20 °C until their use as template in PCR assays.

Touchdown PCR assay

In this study, a polymerase chain reaction (PCR) assay targeting a transposon-like repetitive region of the bacterial genome (IS1111 gene) was used to detect *C. burnetii*. The primers trans-1 (5_ -TAT GTA TCC ACC GTA GCC AGT C-3_) and trans-2 (5_-CCC AAC AAC ACC TCC TTA TTC-3_), were used which were synthesized by Bioneer Co. (South Korea). These primers amplify a 687-bp fragment of the target sequence. Touchdown PCR assay was performed as described previously (Hoover *et al*., 1992). The PCR mixture (25 µl) included 2.5 µl of 10× PCR buffer (100 mM Tris-HCl buffer, pH 8.3, 500 mM KCl, 15 mM MgCl2, and 0.01% gelatin), 200 µM deoxynucleosidetriphosphate mix, 2 µM of each primers, 0.3 U of Taq DNA polymerase, 3 µl of template DNA, and high pure double sterilized water to make up the reaction mixture volume. Touchdown PCR assay was performed in a personal thermocycler (Techne, England) under the following conditions: denaturation of DNA at 95°C for 2 min, followed by five cycles at 94°C for 30 s, 66 to 61°C (the temperature was decreased by 1°C between consecutive steps) for 1 min, and 72°C for 1 min. These cycles were followed by 35 cycles consisting of 94° C for 30 s, 61°C for 30 s, and 72° C for 1 min and then a final extension step of 10 min at 72°C.

Detection of amplified product

Amplimers were resolved on 1% agarose electrophoresis carried out in Tris acetate EDTA buffer for 60 min at 120V and stained with ethidium bromide (0.4 mg mL-1 for 20 min) (Fig 1).

Sequence analysis

The first positive PCR product was purified using the Roche purification kit (Roche Molecular Biochemicals, Mannheim, Germany) and submitted for automated sequencing in both directions at the Eurofins MWG Operon (Martinsried, Germany) using PCR primers as sequencing primers. Nucleotide and predicted amino acid sequence data were aligned with the Clustal alignment algorithm. Phylogenetic analysis based on nucleotide sequences was conducted using a distance method, unweighted pair group with arithmetic mean, by calculating boots trap values for 1000 replicates in CLC main Workbench Package Version 5 (CLC Bio, Aarhus, Denmark).

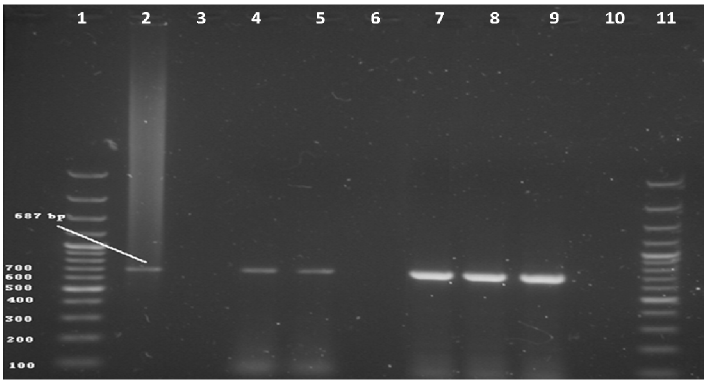


Figure 1. Detection of *C. burnetii* in bulk tank milk samples by touchdown-PCR assay, amplifying of 687 bp segment of IS1111 gene: lanes-1&11:100 bp DNA ladder; lane-2: positive control, lane-3: negative control; lanes 4,5,7,8,9, positive samples.

Results

After DNA extraction and performing the touchdown PCR assay, 5 out of 100 (5%) bovine bulk tank milk samples detected as positive. The 687 base pairs of the amplified

IS1111 gene fragment were successfully sequenced from one of the PCR-positive samples. No differences were found in nucleotide and deduced amino acid compared to published sequences of *C. burnetti* in Gene bank. The sequence (JKJB100IR) was deposited in GenBank and assigned the following accession number KF146935.

Discussion

C. burnetii is the agent of a common bacteriosis between human and animals (Kim *et al.*, 2005). Cases of acute Q fever have been reported in human, mostly in spring and early summer. The disease may occur at all ages, but they are more frequent in men than women (Raoult, 1990). Individuals who come into contact with farm animals including the veterinarians, farmers, laboratory staffs and slaughter plant personnel are more at risk of infection, and the prevalence of 7% to 45% is reported among these people (Seyitolu *et al.*, 2006, Cetinkaya *et al.*, 2000).

C. burnetii infection in dairy herds can led to shedding of microorganism through other ways including, feces, urine, vaginal discharge, embryo fluids and fetal tissues such as liver and spleen, which are important sources of infection for human (To *et al.*, 1998). Although pasteurization of milk and controlling the hygienic principles during milk processing in Iran is being recommended for many years, but non-pasteurized milk and dairy products such as cheese are quite common. In fact consuming fresh and non-pasteurized milk is common in some rural areas. In this study 100 samples of milk which were taken from bulk tank milk of dairy farms in northeastern of Iran were evaluated for the presence of *C. burnetii*. During the spring of 2012, sampling was performed directly from bulk tank milk of dairy herds.

It should be mentioned that the shedding route of bacteria varies in different species of ruminants. Rodolakis *et al.*, (2007) studied shedding route of *C. burnetii* in ruminants and reported that *C. burnetii* shedding in sheep is mainly through vaginal discharges and excretions, in goat through milk, vaginal discharges and excretions, whereas in dairy cattle the shedding is predominantly via milk (Rodolakis *et al.*, 2007).

In order to identification of *C. burnetii* in milk and dairy products the PCR method is a safe and useful method. In contrast, isolation of *C. burnetii* is hazardous, difficult, and time-consuming. Besides the isolation of this microorganism must be performed in biosafety-level 3 laboratories (Arricau-Bouvery and Rodolakis, 2005, Barlow *et al.*, 2008)

In the present study, to determine the shedding of the microorganism, milk samples were taken randomly without considering the health of the animals, but there are some reports on association between *C. burnetii* shedding in milk and metritis, mastitis and abortion (Muskens *et al.*, 2011). Moreover, infected animals shed the bacteria intermittently through their milk (Kim *et al.*, 2005), thus, identifying *C. burnetii* in milk depends mainly on the time of sampling, therefore testing animal based on only milk sample can be lead to misclassify the status of the animal infection (Guatteo *et al.*, 2006). In the present study, using touch-down PCR assay targeting the IS1111 gene by Trans-1 and trans-2 primers , 5% of bulk tank milk samples were detected as positive for *C. burnetii*.

Considering that the bulk tank milk has not undergone any heating process, and regarding the high resistance of microorganism in environmental conditions, the positive cases in PCR test can be presumably related to the living microorganism, although PCR test is not capable of separating target living cells from dead ones. Other studies have reported a wide range of the presence of this microorganism in milk, 6.2% in bovine bulk milk, from central area of Iran (Rahimi *et al.*, 2010), whereas 83.8% of bovine milk from France (Berri *et*

al., 2000), 53.7% from Japan (Maurin and Raoult, 1999) and 14.3% from Italy (Ongor *et al*., 2004).

Fretz *et al*., (2007), used nested-PCR assay for the identification of *C. burnetii*; all 81 samples of sheep bulk tank milk and 39 goat bulk tank milk was reported as negative, while from 359 cattle bulk tank milk 17 samples (4/7%) were positive (Fretz *et al*., 2007).

In another study in Iran for the identification of *C. burnetii*, nested-PCR method was used, on 110, 56 and 210 samples of bulk tank milk of sheep, goat and cattle. All sheep samples were negative, whereas 13 cattle samples (6.2%) and only one (1.78%) goat sample reported as positive (Rahimi *et al*., 2010).

Touchdown-PCR method has been used in this study, because higher annealing temperatures in initial few rounds gives greater specificity, and the lower temperatures in later stage permit more efficient amplification from the specific products formed during the initial cycles (Schiavoni *et al*., 2010).

The 687 base pairs of the amplified gene fragment were successfully sequenced from one of the PCR-positive samples and by comparing to published sequences of *C. burnetti* in Gene bank, no differences in nucleotide and deduced amino acid were found. It has been reported that, the detection limit for the *C. burnetii* in PBS was 10-fold higher than that in milk (Muramatsu *et al*., 1997). In this study for preparation of PCR mixture and excluding the inhibitors which might be present in raw milk, samples were centrifuged three times and each time the pellet were resuspend in PBS.

According to our findings that shows the presence of this microorganism in bulk tank milk samples, it is recommend that donot consuming unpasteurized dairy products, and also monitoring the time and temperature of milk pasteurization as an important critical control point. .Furthermore, further studies is needed to determine the prevalence of the infection in dairy herds in this area.

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شناسائی کوکسیلا برنتی و تعیین توالی ژن *IS1111* در شیر مخزن گله های گاو شیری

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چکیده

کوکسیلا برنتی عامل بیماری تب Q درجوامع انسانی است. این مطالعه با هدف تعیین میزان آلودگی نمونه های شیر مخزن به این باکتری انجام گردیده است. در این مطالعه تعداد ۱۰۰ نمونه شیر مخزن جمع آوری شده از گله های شیری در شمال شـرق ایـران بـه روش touchdown PCR مورد بررسی قرار گرفت. جهت تکثیر قطعه ۶۸۷ جفت بازی از ژن *IS1111* پرایمر های ترنس مورد استفاده قرار گرفـت. تعـداد پـنج نمونـه (۵٪) از نظر حضور باکتری کوکسیلا برنتی مثبت تشخیص داده شد. نتایج تعیین توالی مشابهت ۱۰۰٪ محصول تکثیر شده را با تـوالی هـای ثبـت شـده در بانک ژن نشان داد. نتایج این مطالعه مشخص نمود که شیر مخزن گاوی می تواند منبع مهمی برای انتقال عفونت این پاتوژن در ایران باشد.

واژگان کلیدی: شیر مخزن، کوکسیلا برنتی، touchdown PCR